Project Title: Development of a Rapid-Cycle Breeding Tool for Pear

Report Type: Final Project Report

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Project Duration: 3 Year + No Cost Extension

Total Project Request for Year 1 Funding: $ 32,915
Total Project Request for Year 2 Funding: $ 33,737
Total Project Request for Year 3 Funding: $ 68,825

Other related/associated funding sources: Awarded
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Agency Name: USDA-ARS, In-house project
Notes: In-house project with complimentary objectives. Half funding for 100% FTE (salary+benefits) technician for years 1 and 2 ($30,705 and $31,536.50, respectively).

WTFRC Collaborative Costs: none

Budget 1
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Item	2021	2022	2023	2024
Salaries	$22,250.00	$22,850.00	$48,279.00	$0.00
Benefits	$8,455.00	$8,687.00	$18,346.00	$0.00
Wages				
Benefits				
RCA Room Rental				
Shipping				
Supplies	$2,210.00	$2,200.00	$2,200.00	$0.00
Travel				
Plot Fees				
Miscellaneous				
Total	$32,915.00	$33,737.00	$68,825.00	$0.00

Footnotes: 1 Biological Science Technician = Half funding for 100% FTE (salary+benefits) technician for years 1 and 2, and full funding for year 3.
2 Supplies: RNA/DNA extraction, tissue culture, greenhouse, molecular supplies and consumables.
If project duration is only 1 year, delete Year 2 and Year 3 columns.

Objectives

1. **Transform pear rootstock germplasm with a flowering-activating, chemically-induced system.** Introduce flowering genes into fire-blight resistant pear rootstock germplasm whose expression can be induced by an inexpensive agrochemical, allowing early flowering for rapid breeding without the negative phenotypes seen in other Rapid-Cycle Breeding (RCB) systems.
2. **Early molecular and phenotypic characterization of transformants.** Confirm the presence and location of the inducible flower genes. Test lines for flowering response.
3. **In-depth characterization and optimization of RCB plants.** Characterize flowering gene expression and flowering response to agrochemical in detail. Determine optimal dose and delivery of chemical induction. Test viability of flowers to be pollinated and begin crossing with germplasm containing additional traits of interest.

Significant Findings

Major improvements in and understanding of adventitious shoot regeneration in Bartlett, OHxF 87 and OHxF 97. Our findings on plant responses to different mineral nutrients and hormones in the regeneration, micropropagation, and rooting between cultivars are important inputs for the development of nursery protocols for tissue culture-based propagation.

Successful transformation of callus tissue in 3 cultivars. Our success in the initial phases of the transformation process bring us one step closer to introducing tools like rapid cycle breeding, or any other biotechnology-based tools, which will be important for breeding programs and future research leading to the development of new rootstocks.

Built connections and collaboration with Strauss Lab at Oregon State University to test different strains of Agrobacterium that can enhance adventitious shoot regeneration from transformed callus tissue. Similar to the transformation of callus, this will be helpful for introduction of biotechnological tools for more difficult cultivars that don't respond as well to tradition Agrobacterium strains. This has potential to aid the use of more varied cultivars in a rapid cycle breeding system.

Results

Objective 1. Transform pear rootstock germplasm with flowering-activating, chemically-induced system (Years 1-2)

1a. Selection of germplasm to be transformed

In Year 1, we were able to obtain 'OHxF 87', 'OHxF 97' (recently confirmed to actually be 'Old Home' x 'Bartlett' crosses by [1]), and 'Bartlett' tissue and initiated these into tissue culture. Successful micropropagation has continued successfully. In years 3 and 4, contamination events temporarily reduced population numbers, however measures were taken to deep clean spaces and purchase newer equipment when necessary to maintain sterility. Additionally in year 3, we obtained the 'Conference' cultivar, as this has been transformed successfully in other labs, as it is particularly amenable to shoot regeneration, even in the presence of agrobacterium [2]. In year 4, we have begun to use 'Conference' in transformation experiments.

1b. Use developed transgenic flower-inducing constructs and develop additional versions

In year 1, we obtained the original RCB construct from the Cutler lab at UC Riverside, which contained the *FLOWERING LOCUS T (FT)* gene from Arabidopsis, a red fluorescence marker (RFP),

and the necessary proteins to make the flowering gene inducible (Inducibility machinery) (Fig. 1A). We modified the construct to contain an antibiotic resistance gene (*NptII*, conferring resistance to Kanamycin), and one of two flowering genes that have been used for early flowering previously in apples and pears (*CiFT* from citrus, and *BpMADS4* from birch [3, 4]) (Fig. 1B). In year 2 we made an additional version, replacing the Kanamycin resistance gene for a Hygromycin resistance gene, as we had found examples in the literature of varying sensitivities to Kanamycin across plant species (Fig. 1C) [5-7]. In year 4, we sequenced this version of the construct to confirm it is correct, and plan to use it in transformation experiments in the future.

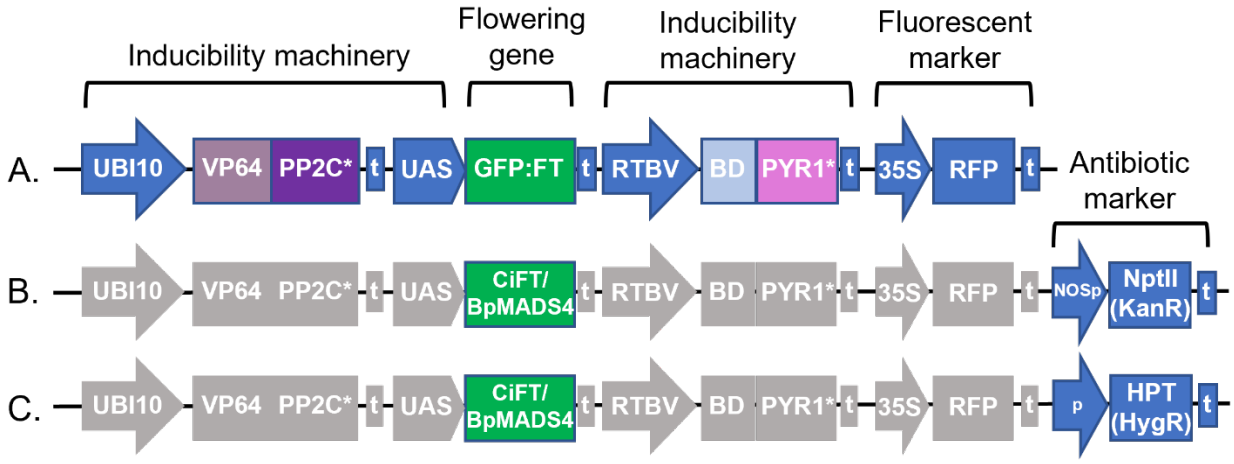

Figure 1. RCB construct development. A. Original construct received from Cutler lab. B. Construct developed in Year 1, containing flowering genes for pear and a Kanamycin-resistance gene (NptII). C. Construct developed in Year 2 containing a Hygromycin-resistance gene (Hpt), replacing KanR.

1c. Transform germplasm

In year 2 we confirmed that the RCB construct was functional and transformable by transforming Arabidopsis and obtaining seeds with the construct inserted (Fig. 2A and B). We further showed that pear callus tissue was successfully being transformed, as indicated by glowing red tissue resulting from the fluorescent marker included in the construct (Figs. 1 and 2C-F). Pear callus is the tissue formed in response to wounding and hormone inputs, and acts as an intermediate tissue from which new adventitious shoots can regenerate, given the ideal hormone inputs and growth conditions. Throughout year 3 we continued transformation trials, altering experimental parameters to improve callus transformation and determine protocols for shoot regeneration from this callus. Our initial base protocol used the following parameters:

Agrobacterium containing the RCB construct was grown overnight until saturation, then diluted in the morning and grown to an optical density of OD600 = 0.8. Growth media contained 100uM acetosyringone to stimulate agrobacterium virulence. Young leaves, just fully expanded, were excised from tissue culture-propagated plantlets and soaked in liquid NN69 media [8] containing and hormones (22uM TDZ as the cytokinin, and 10uM NAA as the auxin) for 60 minutes to avoid oxidative browning and stimulate callus production. Leaves were transferred to media-moistened filter paper and 4mm biopsy punches were used to cut leaf discs from the petiole-end of the leaves (2 leaf discs per leaf), with each leaf disc containing midrib tissue. The biopsy punches introduce wounding around the entire edge of each disc, and we included midrib tissue, as it tends to be more competent to develop callus and adventitious shoots. Leaf discs were moved to inoculation media containing the agrobacterium, acetosyringone, and 30g/L sucrose, and left to soak for 60 minutes. Control leaf discs were soaked in identical media without agrobacterium added. Leaf discs were then moved to liquid co-cultivation NN69 media containing 30g/L sucrose and hormones (22uM TDZ and

10uM NAA) and kept in the dark for 4 days at 20C, to allow growth of both the agrobacterium and the callus tissue. After 4 days, antibiotics were added to the liquid media (300mg/L Cefotaxime and 200mg/L Timentin) and left to culture overnight to eliminate the agrobacterium and prevent overgrowth. Leaf discs were then transferred to solid NN69 media, containing 50mg/L Kanamycin, 30g/L sucrose, and hormones (22uM TDZ and 10uM NAA), and grown in darkness at 20C for 2 weeks. After 2 weeks, plates were moved to unlit shelves, maintained at room temperature, and checked weekly for red fluorescence and adventitious shoot regeneration. Callus transformation, indicated by red glowing spots (# of red spots/total # leaf discs transformed), was reported at 4 weeks, and adventitious shoot regeneration is reported at 8 weeks. Leaf discs were transferred to fresh media every subsequent 4 weeks.

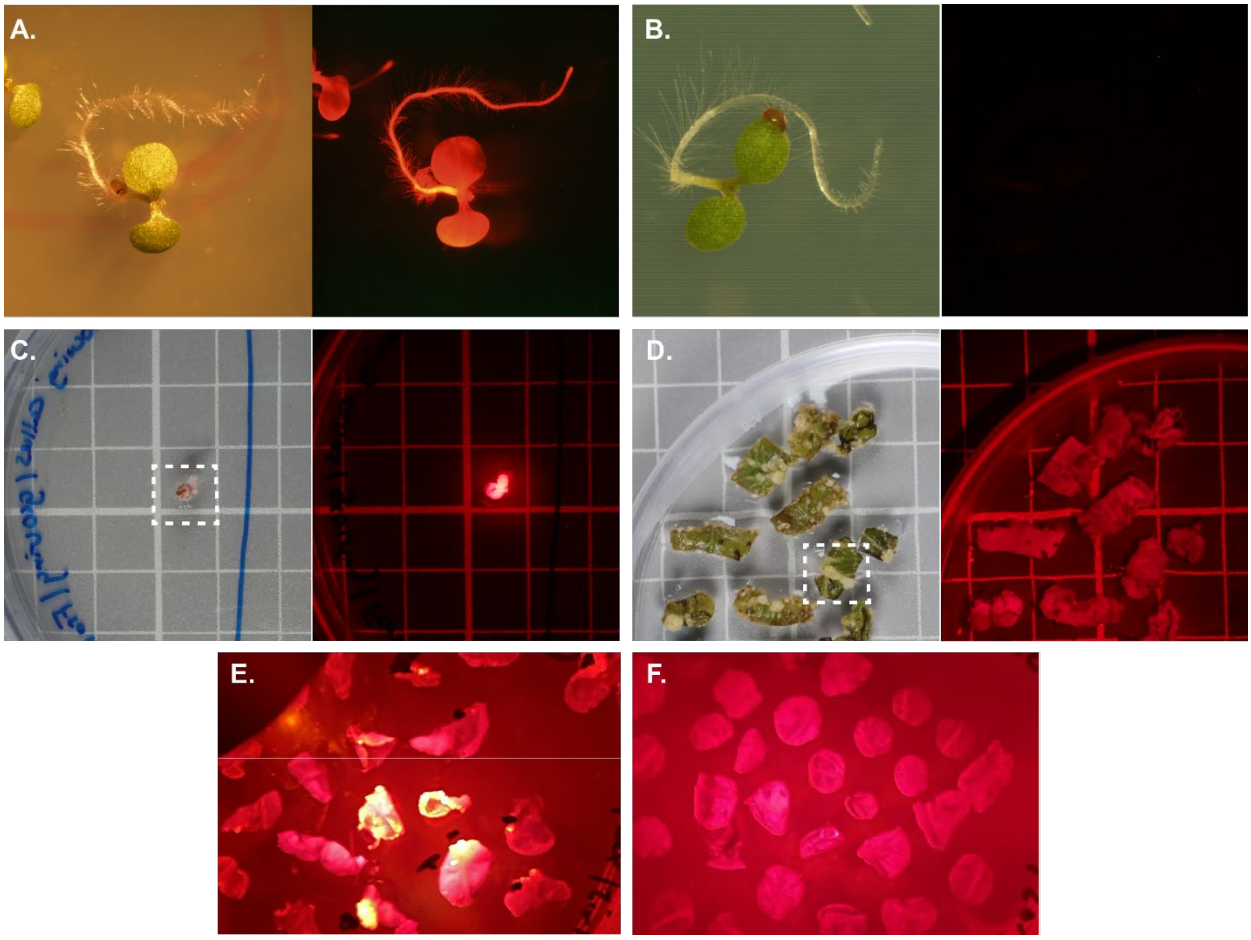

Figure 2. Red fluorescent marker indicates transformation of Arabidopsis and pear callus tissue. A. Arabidopsis seedlings that have been successfully transformed with the RCB construct and selected on Kanamycin, in white light (left) and green light to excite the red fluorescence (right). B. Arabidopsis seedlings that have not been transformed, for reference. Chlorophyll fluoresces to a low level, but the bright red of the fluorescent marker is absent. C. Transformed pear callus that has been isolated from a leaf, in white light (left) and green light (right). D. Non-transformed (control), pear leaf squares growing callus, not showing the bright red of the marker. E-F. Additional images of transformed and glowing callus (left) and non-transformed control callus (right) on leaf discs generated in year 3.

Table 1 contains results from trials throughout years 1-4, where we varied different parameters identified from the literature to be beneficial for regenerating different plant species from transformation events [9-14]. In these trials, we tested leaf tissue wounding methods (slicing whole

ID/ Date	Experimental parameters tested	Cultivars	Callus transform.	Takeaways
211210	First run with protocols from Kearneysville	Bartlett	Unknown	Method of Agrobacterium inoculum prep is difficult to control - switching to sub-culturing.
220328	Agro conc. (OD600=0.3, 0.6)	Bartlett	Unknown	No regen
220519	Agro conc. (OD600=0.1, 0.3)	Bartlett	Unknown	No regen
220614	Inoculation (soak+cut, vacuum)	Bartlett	Unknown	No regen, review of more protocols points to need for AS earlier.
220615	Agro conc. (OD600=.008, 0.3) Inoculation (soak+cut, vacuum)	Bartlett	Unknown	Vacuum infiltration does not seem to be an improvement.
220803	Inoculation (soak+cut, vacuum)	Bartlett	Unknown	Agrobacterium overgrowth shortened experiment - different antibiotics needed.
221028	Tested multiple Kanamycin-Resistance-containing vectors, including RCB construct	Bartlett	Unknown	Regeneration of controls (without Agro) is improved on NN69 media. Antibiotics helped with knocking down overgrowth.
221110	Agro conc. - tested OD600=0.8 Tested multiple vectors again	Bartlett	Unknown	One leaf formed and later died, but briefly had a transformant. Still some issues with Agro overgrowth.
221205	Using biopsy punches Agro removal - testing pre-selection	Bartlett	65/150= 0.43	Biopsy punches improve time and get similar callus growth.
221215	Same as RCB_221205	Bartlett	35/173= 0.23	
230315	Carbohydrate source - sucrose vs. sorbitol	Bartlett	Suc. 22/50= 0.44 Sor. 14/49= 0.29 1:1 15/49= 0.30	No significant difference in callus transformation with different carbohydrate sources.
230316	Cultivar type, protocol same as 221028 and 221110	OHxF97	97 62/101= 0.61	New standard protocol yields highest rates so far.
230622	Cultivar type, protocol same as 221028 and 221110	Bartlett OHxF87	B 56/90= 0.62 87 53/85= 0.62	New standard protocol yields highest rates so far.
230714	Pre-culture callus before inoculating	Bartlett	0/75= 0	Callus growth prior to inoculation appears to block transformation.
230928	Microprop. Media (DKW, PM2, QL)	Bartlett	n/a	Lost to fungal contamination
231016	Microprop. Media (DKW, PM2, QL, varying CK)	Bartlett	D+BA 64/55= 1.2 Q+mT 54/69=0.78 Q+BA 22/56=0.39	DKW media with BA as cytokinin and QL media with mT give highest rates.
241007	Following protocol from NZ group	Conference Bartlett OHxF 97	n/a	Published protocols must be missing info. Leaves did not produce callus.
241108	Following protocol from NZ group, using LBA4404 Agro	Conference Bartlett	n/a	Bacterial contamination and no callus growth.

Table 1. Experimental comparison, outcomes, takeaways from standard transformation trials. Abbreviations: AS – acetosyringone, NN69 – Nitsch and Nitsch 69 media, MS – Murashige and Skoog media, DKW – Driver Kuniyuki Walnut media, Q or QL – Quoirin and Lepoivre media, mT – meta-Topolin (cytokinin), BA – 6-Benzylaminopurine (cytokinin).

leaves vs. cutting into squares or circles with biopsy punches), leaf tissue soaking to avoid oxidation (with and without hormones included), liquid versus solid media during the co-cultivation stage, Agrobacterium concentration and strain, carbon source (sucrose vs. sorbitol), multiple different hormone combinations, micropropagation media prior to transformation, and testing published protocols from other labs. Thus far, we found that for 'OHxF87' and 'OHxF97', our current standard protocol (written above) resulted in the highest amount of transformed callus, determined by the number of red fluorescent spots in leaf callus, and for 'Bartlett', this protocol combined with plant growth on DKW media prior to leaf excision worked best (Table 1).

In year 4, we began conducting transformation trials using an additional strain of Agrobacterium, obtained from the Strauss lab at Oregon State University [15]. This Agrobacterium strain, called S82, has been used in combination with standard Agrobacterium (which contains the construct of interest) to enhance transformation rates of very difficult-to-transform cultivars of eucalyptus and poplar [16]. Briefly, the S82 strain contains phytohormone-biosynthesis genes (for plant auxins and cytokinins) that a wild strain of Agrobacterium would have, meaning that cells transformed with S82 are able to biosynthesize these hormones and signal to surrounding cells to divide and grow. When the S82 strain transforms plant cells, those cells divide and grow into callus tissue, but never appear to regenerate their own adventitious shoots. Instead, they signal to surrounding callus tissue to regenerate. Thus, when plant tissue is transformed with both S82 and Agrobacterium containing a construct of interest, like our RCB construct, the S82-transformed callus should signal to the nearby RCB-construct-transformed callus to develop adventitious shoots. Additionally, the S82 cells have a green fluorescent marker, so we can track the number and location of transformed callus (Fig. 3). Table 2 contains results from trials with S82 and Agrobacterium containing our RCB construct.

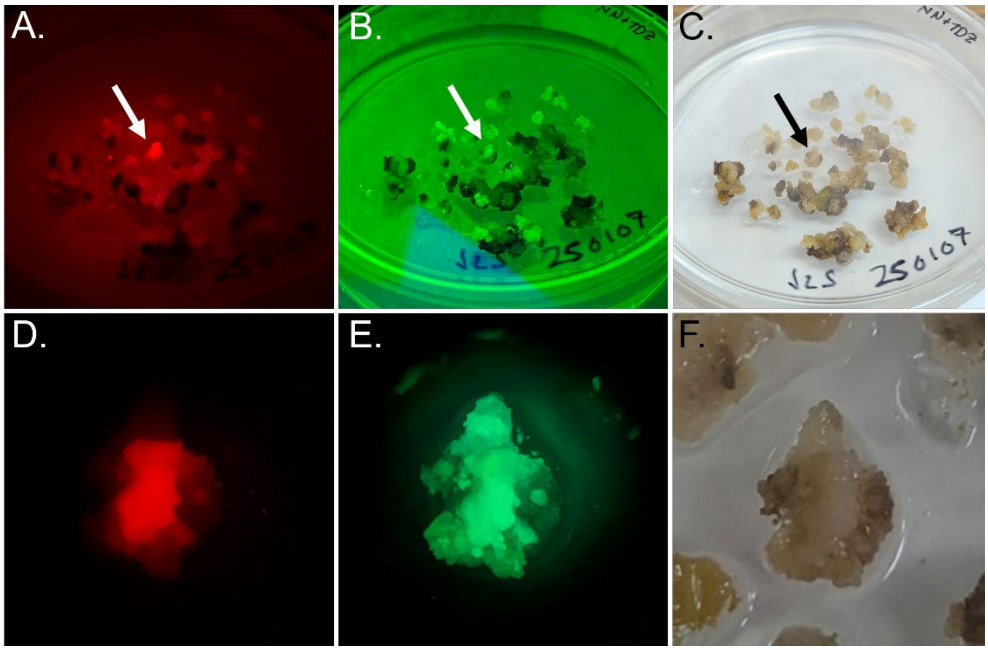

Figure 3. Callus transformed with S82 nearby to callus transformed with RCB construct. A-C. A plate of callus developed from leaf discs inoculate with S82- and RCB-construct-containing Agrobacterium, shown with red fluorescence (A.), green fluorescence (B.) and bright field (C.). Arrow indicates callus zoomed in on in D-F. Green fluorescent marker indicated cell transformed with the S82 strain, and red indicates cells transformed with the RCB construct.

For transformation, we follow a similar protocol to before, but with the following modifications: Excision, soaking, and wounding of leaves remains the same. Leaves are inoculated in a ratio of

RCB-containing to S82-containing agrobacterium for 20 minutes and then co-cultivated on solid Nitche & Nitche 69 (NN69) media for 5 days at 25C under dark conditions. Leaf discs are washed with sterile milli-Q water 3 times followed by a single wash in Nitche& Nitche 69 containing 200 mg/L Timentin, 300 mg/L cefotaxime, 50 mg/L carbenicillin and 600 mg/L Rifamcin (TCRcarb) then cultured on Nitche & Nitche69 agar plates containing 2% sucrose, TCRcarb antibiotics with no hormones for 7 days 'rest' period at 25C under dark conditions. After 7 days leaf discs are cultured on plates containing NN69 but with the addition of 50 mg/L kanamycin in addition to the TCRcarb antibiotic cocktail at 25C in the dark for 2 weeks then moved into the light, as the S82-transformed callus tissue will produce the hormones needed for adventitious shoot regeneration. Leaf discs are checked for fluorescence and adventitious shoots every 2 weeks after being moved to 16:8 light conditions.

Following this protocol, we have tested multiple different ratios of S82:RCB-construct-containing Agrobacterium (25:1, 10:1, 5:1, 2.5:1, and 1:1). We found that 2.5:1 gives the highest numbers of callus transformation (Table 2). Further, we have varied the "rest" period, which is the period after co-cultivation that leaf discs are allowed to grow on media without Kanamycin, to allow callus containing S82 to grow. We found that having no rest period results in no callus transformation, while a period of 7 days allows for substantial S82-callus growth. In our most recent trial, we are adding no Kanamycin in the initial phase until regenerant form, then we will move these onto Kanamycin plates and look for red fluorescence.

ID/ Date	Experimental parameters compared/tested	Cultivars	Agro. Ratio RCB/S82	Callus transform. RCB	Callus transform. S82	Regen
240119	Comparing ratios of Agro containing RCB construct and S82	Bartlett	1:1 5:1 10:1 25:1	1:1 22/95= 0.23 5:1 40/95= 0.42 10:1 22/80= 0.28 25:1 4/107= 0.04	1:1 49/95= 0.52 5:1 52/95= 0.55 10:1 0/80= 0.00 25:1 3/107= 0.03	22
240206	Comparing ratios, removed "rest period"	Bartlett	5:1 10:1 25:1	n/a	n/a	n/a
240508	Focus on 5:1 ratio with "rest period" returned	Bartlett	5:1	20/107= 0.19	40/107= 0.37	29
241028	Microprop. Media (MS and QL for Bartlett, WPM for OHxF 97, all with 5uM mT)	Bartlett OHxF97	2.5:1	MS 93/74= 1.25 QL 40/47= 0.85 WPM 46/68= 0.68	MS 198/74= 2.68 QL 162/47= 3.45 WPM 196/68= 2.88	1

Table 2. Experimental outcomes from S82 transformation trials. Callus transformation here is reported as the number of callus cell clusters fluorescing from the red or green marker (indicating cells are transformed with the RCB construct or S82, respectively), over the number of total leaf discs in the experiment. The "Regen" column refers to the number of adventitious shoots regenerated, none of which contained the RCB construct at the time of this report. In the first 3 trials, all plants were micropropagated on QL media with 5uM meta-Topolin as cytokinin.

While we have not yet confirmed any adventitious shoots transformed with the RCB construct (only callus thus far), we have gotten adventitious shoots that are not transformed (Regen, Table 2). This is to be expected, as there is plenty of callus growing that has not been transformed and does not glow with either the red or green fluorescent marker. This also signifies that the S82-transformed callus cells are behaving as they should and sending signals to surrounding callus to regenerate into shoots, we just have not yet had enough RCB-construct-containing callus cells near enough to the S82-

containing cells to receive substantial signal. Future trials will focus on trying to increase RCB-construct-containing cells to improve these chances.

In year 4, we have also tested transformation of our RCB construct into 'Conference', using published protocols designed to work for this cultivar specifically [2, 17]. While 'Conference' is not our target germplasm, it has been identified as a cultivar that is easy to regeneration and amenable to transformation [13, 18], making it a good control. In our initial attempt, we followed the protocol outlined in the recent Tomes et al. 2023 paper [17], but we saw no callus transformation (Table 1). This was in part due to bacterial contamination, likely endophytes coming from the plants themselves. However, before bacterial contamination became a major issue, we also found that callus developed was quite slow or not at all, suggesting that the protocols also did not contain all necessary details. We are currently initiating another transformation attempt with 'Conference', following protocols modified by our collaborators in Kearneysville, which have been successful previously with this cultivar.

Objective 2: Early molecular and phenotypic characterization of transformants (Year 2-3)

2a. Rescue transformants, confirm presence of construct

In year 3, we attempted to regenerate plant tissue from the callus that has been transformed. Early in the year we had one regenerant with a red fluorescent-glowing leaf, however this regenerant appeared to have lost the cells containing the shoot apical meristem tissue, and thus never continued to grow. In addition, we found several regenerants that continued to grow on Kanamycin (RCB_230622, Table 1), suggesting they contain the transgene, but their tissue does not glow red when we looked at fluorescence. However, continued growth on Kanamycin led to these regenerants dying. In the future, regenerants that show positive PCR results will be sequenced to confirm the location of the transgene within the genome. Confirmed plants that reach sufficient size will be rooted, acclimated, and moved to soil before moving on to characterization. While we were previously concerned about ability to root these cultivars, in year 2 we tested rooting protocols and saw success for 'Bartlett', 'OHxF 87', and 'OHxF 97'.

2b. Test flowering-induction in response to chemical induction and select clones to move forward

Among transformed plants, we want to initially determine clones that are responsive to chemical induction of flowering. Plants will be sprayed with Mandipropamid and flowering will be observed. These initial flowers will also be analyzed for morphology. Results will be used to determine which transformed lines to move forward with in-depth characterization. Lines will also be replicated/propagated to ensure we have sufficient material for analysis. While we were not able to reach this objective thus far, we hope that this subobjective will begin to be addressed in the coming year.

Objective 3: In-depth characterization and optimization of RCB plants (Year 3+)

3a. Determine gene expression and flowering responses to chemical-induction

While we have not reached this objective yet, when we have confirmed transformed plants, our plans are as follows: Confirmed transformed plants will be allowed to grow until branches can support fruit weight. At this point we will characterize flowering gene expression and flowering responses to chemical induction in more detail. After spraying leaves with Mandipropamid, we will collect leaf and bud tissue and use quantitative PCR to determine gene expression levels compared with control genes and control tissues. We will observe timing of flowering as well as inflorescence and flower morphology. In citrus, the Cutler lab and collaborators have seen high levels of gene expression in response to chemical induction, as well as flowering occurring in the axillary bud associated with

leaves sprayed after about 2-3 weeks. We will perform experiments to determine the optimal chemical doses (varying concentrations), the best way to deliver the chemical (varying addition of surfactant/wetting agents), and how timing of flowering and flower morphology respond to these different factors. Given difficulties in regenerating plants from transformed tissue (Obj. 1c), this work may begin towards the end of coming year.

3b. Test the ability of induced flowers to be pollinated, develop fruit
In other RCB systems, continuous flowering often led to abnormal flower morphology, however in most cases flowers were still able to develop fruit and viable seed. While we hope to avoid these abnormal phenotypes with an inducible system, it will be important to test transformed germplasm to determine whether flowers are able to be pollinated, as well as phenotype fruit and seed development. Further, we expect that with more normal phenotypes, we will be able to identify more lines in a shorter amount of time that have functional bud and flower development. We will induce multiple flowers per plant and observe stages of pollination, fruit set, fruit and seed development, and seed viability. In citrus, these tests were able to be performed in 1 year old transformed trees. This work will take place once we induce and characterize flowers, in Obj. 3a.

3c. Begin crossing with germplasm containing other desirable traits.
Once stable lines have been optimized and characterized, we will begin performing crosses with desirable germplasm. Initially, we will cross with fire-blight resistant germplasm identified in Objective 1a, containing additional sources of resistance to OHxF backgrounds. Because there are multiple sources of fire-blight resistance [19-21], we can perform multiple crosses to introgress fire-blight resistant traits. Future crosses include germplasm identified by the breeding program to show dwarfing traits, or accessions exhibiting resistance to other key pathogens or pests. This tool may also be of use to quickly generate mapping populations for identifying unknown genetic sources of desirable traits.

Future steps beyond the length of this proposal will be phenotyping for fire blight resistance, as well as other traits we may be crossing for. Whenever possible, we will used developed markers to assist in more rapid assessment of traits.

Discussion

Breeding for tree fruit crops can be a very lengthy process, in part due to the length of the tree's juvenility period, which is the time it takes for the tree to bear fruit. In European pears, this can take up to ~10+ years, depending on cultivar. Our goal is to develop a system in pears that shortens the length of this process and allows for more rounds of breeding crosses in a shorter time. In other tree crops, including apple, plum, and citrus, rapid cycle breeding (RCB) systems have been developed and used to drastically shorten this time by introducing flowering genes, typically through overexpressing them. While ultimately useful, overexpression of flowering genes can lead to many abnormal phenotypes, such as early bud termination and weak branches, and often many lines need to be screened to find RCB transformants that are viable. Here, we are trying to introduce an inducible flowering gene, which will allow us to have normal plant phenotypes until flowers are needed, and hopefully decrease the number of plant lines needing to be screened to avoid phenotypes like early termination.

Here we report continued successful transformation of pear callus tissue (Fig. 2). As mentioned above, pear callus is the intermediate tissue that develops in response to wounding and hormones, and from which adventitious shoots can regenerate, given the optimized conditions. It is well understood in the literature that adventitious shoot regeneration in response to hormone inputs is highly cultivar-dependent. While callus transformation has been successful, we have yet to regenerate shoots from

this callus. This tells us that we have identified conditions and hormones that allow for cells to be transformed by Agrobacterium carrying our RCB construct, which is encouraging. This also tells us that we have yet to find conditions in which adventitious shoot regeneration occurs in our cultivars of interest. It is common that rates of regeneration after transformation are much lower than regeneration rates in total absence of Agrobacterium, however it is not well understood why this occurs. Since starting this project, other groups have had success with transforming other cultivars, such as 'Conference', underlining the cultivar-specificity of plant responses to transformation and regeneration treatments. In addition to development of important tools for breeding, our work in understanding the difference between cultivar responses will be very useful for future work in applying biotechnology to more cultivars. Further, our findings may lead to improved predictability for regeneration, transformation, micropropagation, and rooting across cultivars in the future.

Over the timeline of this grant, we were also able to improve callus transformation efficiency in 'Bartlett', 'OHxF 87' and 'OHxF 97'. We have developed a base protocol that when applied to 'OHxF 87' and 'OHxF97', has reached callus transformation efficiencies of 0.62 and 0.61 red fluorescent spots/total leaf discs, respectively (230316 and 230622, Table 1). These efficiency calculations were very similar to 'Bartlett' under the same conditions (230622, Table 1). 'Bartlett' could be additionally improved by growing plants on DKW media supplemented with 4.4uM BA (231016, Table 1), which we have not yet applied to the OHxF cultivars. This was very encouraging, as it suggests that the transformation of cells is working well and similarly for all cultivars tested. This further underlined that adventitious shoot regeneration from transformed callus tissue seems to be the step that varies more widely between cultivars and remains the bottleneck.

We were also able to identify parameters that did not have strong effects on callus transformation in these cultivars. Carbon source, comparing sucrose, sorbitol, and a mixture of the two, showed no significant differences in callus transformation, and did not lead to adventitious shoot regeneration from transformed callus, despite reports that it improve regeneration in 'OHxF333' (cite). Further, based on communications with collaborators at UC Davis, we tested whether pre-culturing callus would improve transformation and regeneration (230714, Table 1), and found that no callus was transformed. We hypothesize allowing the callus to form ahead of time may have actually blocked the Agrobacterium from entering the cells. Early on, we found no difference in the effect of inoculation methods (whether soaking and cutting leaves with a scalpel or vacuum infiltration them in the Agrobacterium inoculum) on adventitious shoot regeneration. However, we did not have a method to check for the red fluorescent marker at the time, so the effect on callus transformation is unknown and worth investigating further.

Late in 2023, a New Zealand group reported development of an RCB tool in 'Conference' pear (cite Tomes). They used a construct that overexpresses the MADS4 flowering gene from Birch (BpMADS4) that has been used to develop these tools in apples (cite Malnoy). The 'Conference' cultivar has been cited in the literature as very easy to regenerate (cite that paper without hormones), which makes it a good starting point, however it is not target germplasm for rootstock research. They also used a different Agrobacterium strain, LBA4404, which we began a trial with, however we faced bacterial contamination and will need to make another attempt (Table 1). We have attempted to follow their protocol exactly as published to transform 'Conference' (as a control), 'Bartlett', and 'OHxF97', however leaves did not produce callus and eventually died. This may suggest the protocol is missing key information to reproduce results, or that other unknown metadata (i.e. water quality, light spectra, etc.) are influencing growth. As mentioned earlier, our next steps are to compare this protocol to the protocols used successfully in Kearneysville for work with 'Conference' and determine if we can transform it as a proof of principle. This would also help us determine whether our difficulties with adventitious shoot regeneration in 'Bartlett', 'OHxF87' and 'OHxF97' are due to cultivar-specific differences.

Recent work with the S82 Agrobacterium strain shows an increase in callus transformation with both constructs, as well as adventitious shoot regeneration, although this is so far from tissue that hasn't contained the RCB construct. This is very promising and we feel that we are very close to transforming these cultivars. We also plan to use the 'Conference' cultivar in future S82 trials, as the literature suggests it is quick to regenerate adventitious shoots. This would further demonstrate cultivar-specific differences, and help us to better understand, and hopefully work toward predicting, what causes these different responses.

Throughout the timeline of this project thus far, we have successfully built a strong tissue culture program in the lab, and have learned much about micropropagation and rooting, in addition to transformation and adventitious shoot regeneration. As stated throughout the report, there are many, many variables that can be altered and tested to optimize each one of these processes. In addition, we have learned a great deal about the need for controlled environmental inputs. In Year 1 and part of 2, plants were grown the in lab, and we found that pear cultures are far more sensitive to the fluctuating ambient temperatures of our building than our colleagues' apple cultures. Purchase of controlled growth chambers aided greatly in growth consistency. In Years 3 and 4, we experience large scale contamination events, due to the failure of cold room near the lab, which when warmed up, grew a large amount of mold. We were able to decontaminate, repaint, and replace the condensers, and this reinforced the need for our sterile spaces to be rearranged, which we have now done. Finally, we learned the scale and time commitment of this types of work, and the need for personnel. Personnel changes, loss, and rehiring presented a largely unavoidable challenge, however this reinforced for us the importance of developing highly detailed and reproducible protocols, such that new lab members can easily learn. We further hope to publish many of these for the use of the community as well.

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Executive Summary

Title: Development of a Rapid-Cycle Breeding Tool for Pear

Keywords: agrobacterium-mediated pear transformation, inducible flowering, RCB

Abstract:

Traditionally, breeding for desirable fruit tree traits is a very long process, in large part due to long juvenility periods, resulting in waiting years for trees to bear flowers for crossing. In several tree crops - for example, apple, plum, citrus, poplar - rapid cycle breeding (RCB) systems have been developed that allow for multiple rounds of crossing within a much shorter time frame, allowing for rapid stacking of desirable traits. We aim to develop an RCB system for European pears, such that rapid breeding can occur to integrate traits like dwarfing (from Dr. Evans' WSU pear rootstock breeding program) and fire blight resistance (connecting with Dr. Bassil's work at the NCGR on Old Home and other genetic sources of resistance), to highlight a few examples. RCB systems in the past have been built on the overexpression of flowering genes, transformed into initial germplasm of interest. However, this constant overexpression of flowering genes can result in undesirable phenotypes, such as weak branches and early termination of flower buds, and require the need to screen many lines to find plants with the right level of expression of flowering. To avoid this, we aimed to transform pears with an inducible-flowering construct, developed by the Cutler lab at UC Riverside and successfully used in citrus. Through the project thus far, we have run numerous trials to transform this inducible-flowering RCB construct into 'Bartlett', 'OHxF 87' and 'OHxF 97'. We included 'Bartlett', as it is common PNW cultivar with a sequenced genome, allowing for future understanding of the genetics underlying transformation. We included 'OHxF 87' and 'OHxF 97', as they have genetics for fire blight resistance and semi-dwarfing. We have had success with transformation of callus tissue, which is the tissue formed in response to wounding and hormone inputs that acts as an intermediate tissue from which new adventitious shoots can regenerate, given the ideal hormone inputs and growth conditions. Further, we have steadily optimized and improved transformation of callus tissue with the RCB construct, which we have been able to monitor through the red fluorescent marker in the construct. However, we have not yet obtained adventitious shoots carrying the inducible-flowering RCB construct. We hypothesize that the 'Bartlett' and 'OHxF' germplasm is more difficult to regenerate and transform that many of the genotypes used in existing literature, due to unknown cultivar-specific requirements. Currently, we are conducting trials with an Agrobacterium strain called S82, which has been used successfully by the Strauss Lab at Oregon State University to transform difficult poplar genotypes by co-inoculation with a construct of interest (in our case, with the RCB construct). We have seen major improvements in callus transformation and are continuing to monitor trials for adventitious shoot regeneration. Once our inducible-flowering RCB construct is transformed into our germplasm, it will be tested for successful inducibility, as well as functional pollination and fruit growth. When these tests are complete, we can use these trees to induce flowering within a year of planting seeds and perform crosses with germplasm of interest to aid in the development of improved rootstocks for the U.S. pear industry.